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13. ABSTRACT (

Tubulin is the major constituent protein of microtubules, which play a critical role in cellular processes like maintenance of cell shape and motility, cell division, differentiation, and maturation. The dinitroaniline herbicide, trifluralin (TF), which has the unique property of binding selectively to plant and algal tubulin, but not to animal tubulin, was recently shown to inhibit both proliferation and differentiation of the plant-like parasitic protozoan, Leishmania mexicana amazonensis. Since the inhibitory effects of TF were shown to be due to its specific binding to leishmanial tubulin and not to host tubulin, we have exploited the selective tubulin-binding property of TF to examine the potential antimalarial effects of this compound. TF, at 1-5 x 10^{-6} M, inhibited (> 98%) the growth and differentiation of Plasmodium falciparum (the most virulent form of human malaria) in culture. Furthermore, there was complete inhibition of in vitro exflagellation of mature microgametocytes (a process which normally occurs in the mid-gut of the mosquito following an infective blood meal), after a 2 hr incubation with TF concentration as low as 10^{-6} M. In addition, ultrastructural studies revealed complete dissolution of the subpellicular microtubule complex of mature gametocytes when treated with 10^{-6} M TF for 2 hr at 37°C, indicating binding of the drug to malaria tubulin. Of particular relevance to this study, the addition of 5 x 10^{-6} M TF to a gametocyte-infected blood meal of mosquitoes, completely inhibited the sexual development of the parasite in the mosquito midgut. Moreover, treatment of early stage gametocyte cultures (Stages II and III) with 5 x 10^{-6} M TF, blocked the in vitro development of exflagellation-competent mature gametocytes (Stage V).

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Anti-Malarial Effects of the Anti-Tubulin Herbicide Trifluralin: Studies with Plasmodium falciparum

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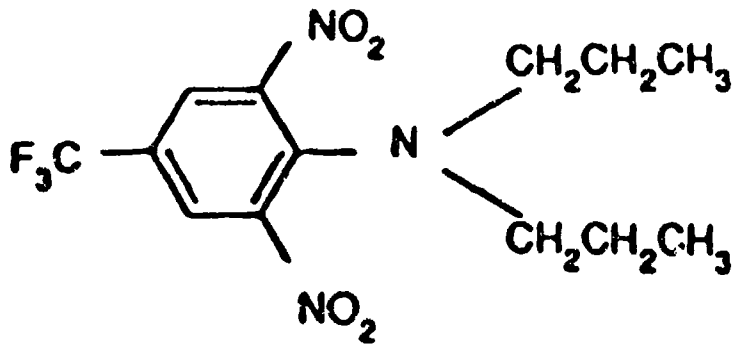
INTRODUCTION

The need for new anti-malarial agents cannot be over-emphasized at a time when the ever-increasing drug-resistance and the lack of an effective vaccine have posed a serious medical threat for both the U.S. military personnel stationed around the world and the millions of civilians in countries worldwide, where malaria is endemic. Malaria, a disease of the blood, necessarily affects the total health of the body. Symptoms are chills, fever, weakness and emaciation. The outcome may be a subclinical condition, recovery, or death. People may react in various ways not only because different species of malaria exist, but because of their general state of health, the treatment they can be given, or the hereditary resistance or susceptibility they possess. The life cycle of human malaria parasites follows a course of development in the blood, mosquito and liver, while the transmission of the disease occurs normally from the bite of an infected anopheline mosquito.

In the present study, we have exploited the selective tubulin-binding property of a novel dinitroaniline herbicide, Trifluralin (α, α, α-trifluro-2-6-dinitro-N, N-dipropyl-p-toluidine) [see Fig 1], that has the unique property of binding to plant and algal tubulin, but not to animal tubulin including mammalian tubulin[1,2]. Tubulin is the major constituent protein of microtubules, which play a critical role in cell motility, cell division and differentiation, intracellular organelle movements, and maintenance of cell shape[3]. The essential role of microtubules in the development, differentiation, and gametogenesis of malaria parasites is well-documented in the literature, and a number of well-studied tubulin-binding drugs have been tested for their anti-malarial effects[4]. However, the same anti-tubulin drugs will adversely affect microtubule-dependent host cell functions as well, and therefore, are of no potential therapeutic value. Recently, Trifluralin was shown to inhibit both proliferation and differentiation of the parasitic protozoan Leishmania mexicana amazonensis[5], which is known to have a number of plant-like attributes[6]. Furthermore, based on the analyses of its DNA sequence, Leishmania tubulin has been reported to be more similar to the trypanosomal and plant proteins than animal proteins[7]. Although the malaria parasites do not have the plant-like features of Leishmania, the rationale for undertaking the present studies was based on the possibility that Trifluralin could bind to malaria tubulin (and not to host tubulin), which would selectively inhibit growth, differentiation and motility of the parasites. By using in vitro blood stage cultures of



Molecular structure of trifluralin

Fig 1

A-1

Plasmodium falciparum, the most virulent form of human malaria parasite, we have observed a very pronounced anti-malarial activity of Trifluralin, which also included transmission-blocking in the mosquito vector. Moreover, in studies in vitro, Trifluralin was found to inhibit P. falciparum gametocyte maturation and viability and to cause dissolution of the characteristic sub-pellicular microtubule complex of mature gametocytes. Details of these studies are described in the present report.

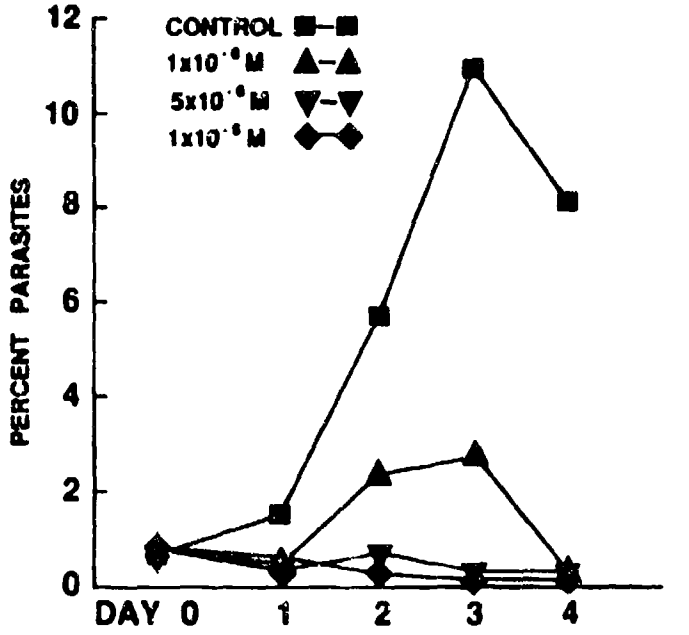
EXPERIMENTAL PROCEDURE

Blood stage cultures of P. falciparum, strain NF 54, were established and maintained following a well-established protocol[8,9]. Various stage-specific malaria parasites were separated by utilizing a discontinuous Percoll density gradient centrifugation method[10], that was modified in our laboratory. Spontaneous exflagellation of matured microgametocytes was induced by exposure to air at ambient temperature[11], and exflagellation centers were monitored and quantitated by either light or phase-contrast microscopy. Parasite growth, differentiation and maturation were monitored by standard procedures using light microscopy of appropriately stained samples. Feeding of infective blood meal to mosquitoes and subsequent follow-up for the development of oocysts in the mosquito mid-gut and sporozoites in mosquito salivary glands, were carried out according to established procedures. For ultrastructural examination of Trifluralin-treated and untreated gametocytes, the parasites were fixed for two hours in 2% glutaraldehyde, pH 7.4, containing 4% sucrose, and the samples were post-fixed in 1% osmium tetroxide for one hour. Electronmicroscopy of ultrathin sections were carried out in Dr. M. Aikawa's laboratory (Institute of Pathology, Case Western Reserve University, Cleveland, Ohio), as previously described[12].

Analytical grade Trifluralin was obtained from Eli Lilly (Dow Elanco), and 1000x concentrated stock solutions were made in dimethyl sulfoxide (DMSO) for various concentrations that were used in the present study. The stock solutions were kept at 4°C in tightly sealed vials, warmed to 37°C before use and added at 1:1000 dilution to the culture samples. DMSO (1:1000) controls were routinely included in all the studies described in the present report.

RESULTS

The present studies, which were designed to examine the anti-malarial effects of the anti-tubulin herbicide Trifluralin, were carried out with blood-stage cultures of P. falciparum, strain NF54, grown in vitro. As shown in Fig. 2, 1-10 μM Trifluralin had a profound inhibitory effect on the growth and differentiation of P. falciparum parasites grown in culture. Almost a complete inhibition (> 98%) of parasitemia was noted in the presence of 5-10 μM Trifluralin. Even at 1 μM concentration of the drug, a marked delay and >75% inhibition of parasitemia was observed. The results shown in Fig. 2 are representative of three separate experiments. Figure 3 illustrates stained



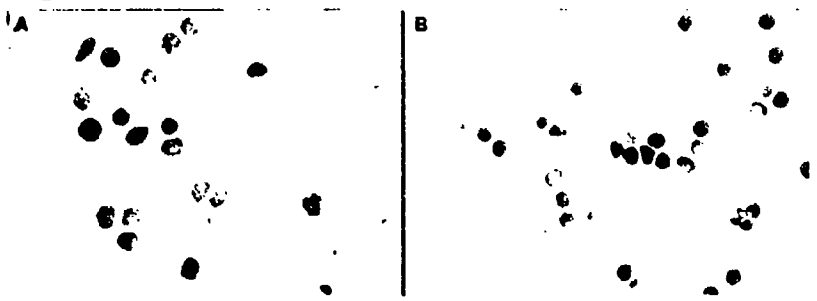
EFFECT OF TRIFLURALIN ON ASYNCHRONOUS CULTURE

Fig. 2

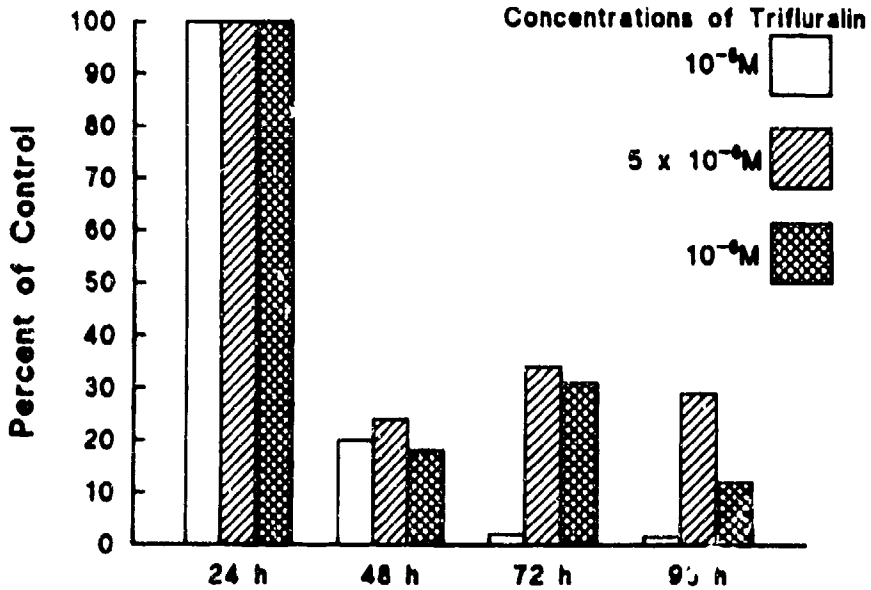
parasite samples which were grown in the absence (panel A) and presence (panel B) of 1 μM Trifluralin for 5 days in culture, and it clearly demonstrates the profound inhibitory effect of the drug.

In order to gain further insight into the mechanism or site of inhibition of parasitemia by Trifluralin, we also examined the effect of Trifluralin on the growth and differentiation of purified stage-specific asexual parasites of P. falciparum. As is shown in Fig. 4, after an initial delay of 24 hrs, a marked inhibition of growth and maturation of early ring stage parasites was observed in the

Fig. 3



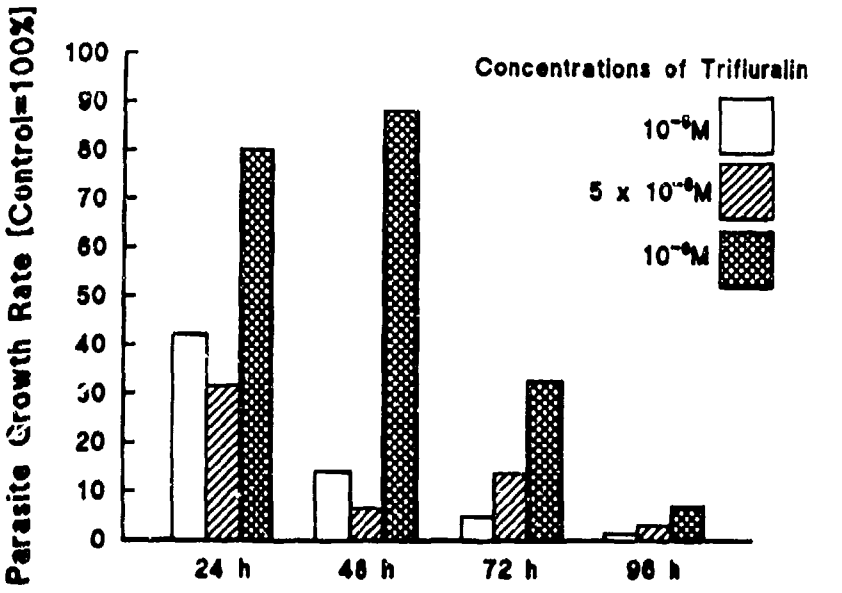
Inhibition of growth and development of blood-stage cultures of *Plasmodium falciparum* by Trifluralin.



Effect of Trifluralin on Ring Stage Parasites

Fig. 4

presence of 1-10 μM Trifluralin and by day 4, this inhibition was almost 100%. Similar inhibition was also noted in the growth and maturation of isolated trophozoite stage parasites (Fig. 5). As the trophozoites are known to be more actively dividing and differentiating than the early ring stage parasites, a significant inhibition of parasitemia was noted even after the first 24 hrs (Fig. 5). This is not an unexpected result, since Trifluralin presumably is binding to the parasite tubulin to cause its growth inhibition, and this protein is known to be an essential functional component of cell division and differentiation.



Effect of Trifluralin on Trophozoite Stage Parasites

Fig. 5

Next, we examined the effect of addition of Trifluralin on the exflagellation of sexually mature malaria gametocytes. Malaria gametocytes do not develop into active gametes in the blood of the vertebrate host, but do so rapidly within the mid-gut of the mosquito (the carrier) or following equilibration of infected blood with air[11]. It is the process of exflagellation that results in the development of several highly motile male gametes that unite with female gametes to initiate the insect stages of malaria. Because the motile machinery of the sexually active gametes is comprised of flagella that are primarily composed of microtubules, and because tubulin is the major protein component of microtubules (to which Trifluralin is going to bind), it was reasonable to expect that Trifluralin's most potent site of action will be during the highly motile process of exflagellation, i.e., the drug will block this process. As shown in Table 1, Trifluralin completely inhibited the spontaneous exflagellation of P. falciparum gametocytes in vitro. The complete inhibition of spontaneous exflagellation, even in the presence of a Trifluralin concentration as low as 10^{-6} M (Table 1), represents one of the lowest effective concentrations of an anti-tubulin drug that has been reported to inhibit a microtubule-dependent cellular process. It should also be noted that a 50% inhibition of exflagellation was observed in the presence of 10^{-9} M Trifluralin as well (Table 1).

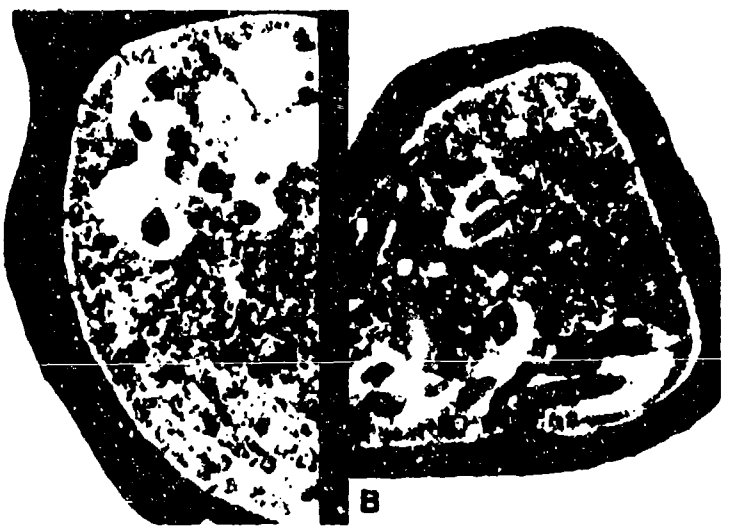
TABLE 1
Effect of Trifluralin on Spontaneous Exflagellation
of P. falciparum Gametocytes

Time (Minute)	Control		Trifluralin Concentration (molar)					
	DMSO (-)	DMSO (+)	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
15-20	+4	+4	+4	+2	-	-	-	-
> 30	+4	+4	+4	+2	-	-	-	-

+4 : > 25 exflagellation centers in 2 sweeps of field
- : no exflagellation observed

Since mature P. falciparum gametocytes possess a well-ordered parallel array of subpellicular microtubules (Fig. 6, panel A: arrows), which are believed to provide cytoskeletal stability and organization to the malaria gametocyte[13], we also examined the effect of Trifluralin treatment at an ultrastructural level. As is shown in Fig. 6, electronmicroscopic examination of mature P. falciparum gametocytes after treatment with 10^{-6} M Trifluralin for 2 hours, revealed a complete dissolution of the

Fig. 6

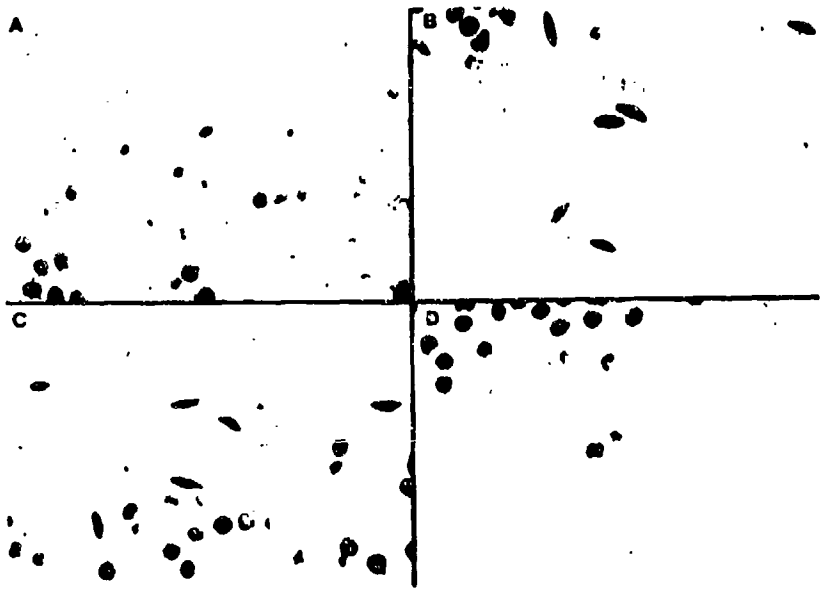


Ultrastructural effects of Trifluralin on
P. Falciparum Gametocytes

characteristic subpellicular microtubule complex of the gametocytes (panel B). These results provide strong supportive evidence for a correlation between the anti-malarial effects of Trifluralin and the drug's selective binding to tubulin/microtubules of the malaria parasite. In contrast, under identical experimental conditions, Trifluralin did not affect either human platelet microtubules or rat neuronal cell microtubules (data not shown). Moreover, in six different experiments, carried out with different parasite cultures containing mature exflagellation-competent gametocytes, the addition of 5 μM Trifluralin to a malaria gametocyte-infected blood meal of mosquitoes caused complete inhibition of the sexual development of the parasite, i.e., there were no detectable oocysts in the mid-gut of mosquitoes and no sporozoites could be found in the salivary glands of the mosquitoes. These results demonstrate the drug's transmission-blocking effect in the mosquito vector and further strengthen the possibility of Trifluralin's usage as an effective anti-malarial agent.

Finally, we also investigated the effect of Trifluralin on the in vitro gametocytogenesis of P. falciparum parasites. As is shown in Fig. 7, when 5 μM Trifluralin was added to an enriched blood-stage culture of P. falciparum containing early stage (Stages II and III) gametocytes (Fig. 7, panel A) and the culture was allowed to continue for additional 6-7 days, during which period the mature exflagellation-competent (stage V) gametocytes develop in culture (Fig. 7, panel C), a complete inhibition of gametocytogenesis was noted in the presence of the drug (Fig. 7, panel D). As shown in Fig. 7, panel B, DMSO (1:1000 dilution), the solvent for Trifluralin, did not have any inhibitory effect on the process.

Fig. 7



Effects of Trifluralin on the growth and maturation of *Plasmodium falciparum* gametocytes.

DISCUSSION

The results of our present study demonstrate the potent anti-malarial effects of Trifluralin when tested in P. falciparum cultures grown in vitro, and also demonstrate the drug's transmission-blocking effect in the mosquito vector. The worldwide increase in drug resistance of malaria parasites has stimulated a search for new anti-malarial agents. The unique tubulin-binding property of Trifluralin and the low effective concentrations of the drug in vitro, offer features that are both novel and advantageous for application of Trifluralin in the development of a new anti-malarial agent of therapeutic value. To our knowledge, this is the first report on the growth-inhibitory effects of Trifluralin on human malaria parasites. The strategies utilized in the present study may also be applied for preventive measures against parasitic diseases in general. Because of the selective tubulin-binding property of Trifluralin, this drug, when suitably administered in an appropriate vehicle/carrier, may provide effective protection against other blood-borne or tissue parasites. Furthermore, Trifluralin is economical and considered safe for man and domesticated animals. The promising results of the present study, together with the selective tubulin-binding property of Trifluralin, clearly warrant further investigations into the potential application of the drug in the treatment of malaria. Successful development of a new anti-malarial agent of therapeutic value will benefit greatly both the U.S. military personnel stationed around the world and the millions of civilians in countries worldwide.

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